Osteoarthritis and Cartilage (2001) **9,** 248–256

doi:10.1053/joca.2000.0382, available online at http://www.idealibrary.com on IDEAL®

1063–4584/01/030248+09 $35.00/0

Osteoarthritis and Cartilage

Journal of the OsteoArthritis Research Society International

High-efficiency non-viral transfection of primary chondrocytes and perichondrial cells for *ex-vivo* gene therapy to repair articular cartilage defects

R. S. Goomer*, L. J. Deftos†‡, R. Terkeltaub†‡, T. Maris*, M. C. Lee*§, F. L. Harwood* and D. Amiel*

**Departments of Orthopedics and †Medicine, University of California, San Diego School of Medicine, 9500 Gilman Dr., La Jolla, California 92093-0630, U.S.A.; ‡Veterans Administration Medical Center, La Jolla, California, U.S.A.; §Dept of Orthopedic Surgery, Seoul National University, College of Medicine, Seoul, Korea*

Summary

Background: Primary perichondrial cells and chondrocytes have been used to repair articular cartilage defects in tissue engineering studies involving various animal models. Transfection of these cells with a gene that induces chondrocytic phenotype may form an ideal method to affect tissue engineering of articular cartilage.

Design: A protocol for high-efficiency transfection of primary perichondrial and cartilage cells was optimized. Plasmids carrying the marker β-galactosidase (β-gal), PTHrP and TGF-β1 genes driven by a strong mammalian promoter were transfected into primary perichondrial cells and chondrocytes. A three-step method was used to achieve high efficiency of transfection: (1) permeabilization of primary cells using a mild detergent, (2) association of plasmid DNAs with a polycationic (poly-l-lysine) core covalently linked to a receptor ligand (transferrin), (3) introduction of cationic liposomes to form the quaternary complex. For *in-vivo* assessment, polylactic acid (PLA) scaffolds seeded with β-gal transfected perichondrial cells were implanted into experimentally created osteochondral defects in rabbit knees for 1 week.

Results: The efficiency of transfection was determined to be over 70% *in vitro*. The transformed cells continued to express β-gal, *in vivo* for the entire test period of 7 days. Furthermore, primary perichondrial cells transfected with TGF-β1 and PTHrP over-expressed their cognate gene products.

Conclusion: The ability to transfect autologous primary perichondrial cells and chondrocytes with high efficiency using a non-viral system may form a first step towards tissue engineering with these transformed cells to repair articular cartilage defects. © 2001 OsteoArthritis Research Society International

Key words: Gene therapy, Tissue engineering, Articular cartilage repair, Transfection, Primary perichondrial cells, Primary chondrocytes, PTHrP, TGF-β1.

Introduction

To date, *ex-vivo* gene therapy in articular cartilage repair has remained a challenge, primarily due to the difficulty of achieving high-efficiency gene transfer into primary autologous cells derived from perichondrium or cartilage tissues with non-viral techniques. Although viral vectors have shown efficacy in transforming primary cells with high efficiency[1–7] and are useful gene therapy tools in life-threatening situations, the use of non-viral methods for gene delivery in non life-threatening situations, such as to repair articular cartilage defects, remains a desirable goal[8]. For instance, adenovirus vectors may induce host immune response[9–15] in some cases, while retroviral vectors require dividing cells for integration[15]. In addition, there is a minor chance that viral vectors may randomly integrate into the host genome, posing risk of neoplastic transformation[15–17]. Therefore, the quest for a high-efficiency gene delivery system for primary autologous cells that would preclude the possibility of an immune response (i.e. non-viral) in non life-threatening conditions such as articular cartilage defects has continued with the use of either liposomes or ligand-polycations (e.g. transferrin-poly-l-lysine). Using cationic liposomes gene transfection of mammalian cells was achieved[18,19] with efficiencies ranging from 1% to 15%[20–23]. The non-viral transfection of primary cartilage cells has been previously attempted, but yielded low efficiencies[24–26]. Use of liposomes did not result in any toxicity *in vivo*[27]. Another method involved the use of transferrin-poly-l-lysine ligand-polycation complex mediated transfection and yielded 7–8% transfection efficiency[28–30]. Increased transfection efficiency was further achieved by the inclusion of poly-l-lysine polycations to the liposome transfection system[31,32], while utilizing transferrin and cationic liposomes high-efficiency transfection of a cultured cell line (NIH-3T3 cells) has been reported[33]. However, to date none of these techniques has been shown to be successful in attaining very high efficiency (>70%) transfection of primary mammalian cells. The development of a technique for the transfection of primary cells is important because only these cells (as autologous

Received 22 September 1999; revision requested 3 December 1999; revision received 15 August 2000; accepted 8 September 2000.

Address correspondence to: Randal S. Goomer, PhD, Dept of Orthopedics, 0630, UCSD School of Medicine, 9500 Gilman Dr., La Jolla, CA 92093-0630, U.S.A. E-mail: rgoomer@ucsd.edu

carriers) could be used to repair[34–37] articular cartilage defects.

Our laboratory has used a protocol for articular defect repair in the rabbit model where cells derived from the costal perichondrium are seeded into a biodegradable polylactic acid scaffold and implanted into an experimentally created full thickness articular cartilage defect in the rabbit femoral condyle. However, the success of this repair is affected by the phenotype of the implanted cells[36,37]. We have shown that the chondrocytic phenotype of cultured perichondrial cells can be enhanced by exogenous TGF-β1[38]. We initiated the present study based upon the premise that cells transfected with an expression vector carrying the TGF-β1 gene would form the most efficient vehicle for the localized delivery of this morphogen into the repair site. In this study we describe a novel method to achieve high-efficiency gene delivery into primary perichondrial cells and chondrocytes. Using this system we delivered a marker gene into primary perichondrial cells and chondrocytes and show that transfection efficiency greater than 70% can be achieved. When these cells were implanted into an experimentally created osteochondral defect in rabbit femoral condyle, the cells continued to express the transfected gene throughout the test period of 1 week.

A TGF-β1 expression vector carrying the TGF-β1 gene downstream of the hCMV promoter/enhancer was transfected into primary perichondrial cells from rabbit ribs and showed increased TGF-β1 specific gene expression. Furthermore, we have initiated studies to determine the functional effects of parathyroid hormone related protein (PTHrP) and its truncated isomers in primary chondrocytes. PTHrP is involved in the regulation of extracellular matrix components and mineral deposition by chondrocytes. PTHrP and parathyroid hormone (PTH) are similar in their biological actions and are recognized by a common PTH/PTHrP receptor. PTHrP has been reported to be localized in fetal cartilage[39], growing rat condylar cartilage[40], and fetal mouse's Meckel's cartilage[41]. PTHrP mRNA was also expressed in chondrocytes located in proliferation zone of tibial growth plate[42], in articular chondrocytes and in periarticular perichondrium[43,44]. The PTHrP receptor was expressed by mature chondrocytes expressing type II collagen but not by hypertrophic chondrocytes expressing the type X collagen[45,46]. A mouse strain deficient in PTHrP showed diminution of chondrocyte proliferation, premature maturation of chondrocytes and accelerated bone formation[47,48]. PTHrP expression lies downstream of a developmentally regulated morphoregulator, Indian hedgehog (Ihh)[44] and TGF-β1[49], and upstream of an apoptosis related protein, Bcl-2[50]. In order to initiate studies on PTHrP mediated effects on chondrocytes, PTHrP (1-87 N-terminal truncated protein and 1-173 wild type isoform)[51] constructs driven by the hCMV promoter/enhancer were transfected into primary chondrocytes using the three-step DTPLL method described here. The PTHrP expression in these transfected cells was determined.

Methods

LIPOSOMES

Cationic liposomes were prepared as follows: equimolar amounts (63 μM) L-α-phosphatidyl-ethanolamine and dimethyldioctadecyl-ammonium bromide were mixed in chloroform, dried as a film and resuspended in 1 ml deionized water. The aqueous solution was sonicated until the desired (50–100 μm) particle size was achieved—observed as compact particles with size slightly smaller than the nucleolus of mammalian cells displayed in a flattened morphology in a culture plate under 900× magnification in a light microscope.

COVALENT BONDING OF LIGAND (TRANSFERRIN) WITH POLY-L-LYSINE

Apo-transferrin was covalently linked to a positively charged polymeric scaffold [poly-l-lysine (70 kDa)] using standard biochemistry protocols that have been previously published[29].

ISOLATION OF TISSUES (PERICHONDRIUM AND CARTILAGE)

Perichondrium from the rabbit ribs was extracted as follows: the New Zealand white (NZW) rabbits (mature 8–10 months old, with closed epiphysis) were sacrificed according to the animal subjects protocols at University of California, San Diego (UCSD), and costal ribs were removed using sterile procedures. Adhering tissue was cleaned away from the ribs with the use of sterile surgical instruments and perichondrium was isolated by breaking the rib and peeling off the perichondrium tissue. Cartilage was extracted as follows: mature NZW rabbits were sacrificed[36–38] as per the UCSD animal subjects' protocol. The knee joint cavity was opened using sterile techniques and the full depth of cartilage tissue was carefully peeled, using a surgical knife, from the femoral condyles and tibial plateaus. The isolated tissues were washed three times in antibiotic containing buffered salt solution (HEPES buffered saline with penicillin/streptomycin (1500 U/ml)).

CELL PREPARATION AND CULTURE SYSTEM (PERICHONDRIAL CELLS)

The perichondrium tissue was incubated overnight at 37°C under sterile conditions in 0.1% collagenase (CLS-2, Worthington Biochemical, Freehold, NJ) in α-MEM (α-modified Earl's medium, GIBCO BRL, Grand Island, NY) containing 0.5 mM L-glutamate and 500 U/ml penicillin/streptomycin supplemented with 10% fetal bovine serum (FBS; Cansera Intern., Rexcale, Ontario, Canada). Cells and tissue debris were isolated away from the media by passage through a sterile 0.45-μm filter. The cells and tissue debris were enzymatically digested with 0.1% hyaluronidase (Sigma, St Louis, MO) and trypsin (Irvine Scientific, Santa Ana, CA) for 1 h. All cell cultures were performed at 37°C with 5.0% CO_2. Utilizing this protocol we obtained 1×10^6 cells from 100 mg wet weight of tissue in less than 48 h.

CELL PREPARATION AND CULTURE SYSTEM (CHONDROCYTES)

Primary chondrocytes were isolated by enzymatic digestion of the cartilage tissue as described previously[52]. Briefly, chondrocytes were released by digestion, by 2 h of incubation with 0.2% pronase (CalBiochem Inc., San Diego, CA) and a subsequent 22 h of incubation with 0.025% bacterial collagenase P (Boehringer Mannheim, Indianapolis, IN) at 37°C under 5.0% CO_2. They were then grown in Dulbecco's modified Eagle's medium/Ham's F12

(DMEM/F12, 50/50, GIBCO BRL) supplemented with 10% FBS and 25 μg ascorbic acid, 50 μg Gentamicin/ml media. After filtration through a nylon mesh 70 μm cell strainer (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ), the cells were resuspended at a density of 4×10^4 cells per milliliter of total media into 10-cm diameter dishes or into 35 mm six-well plates or cell culture slides (Costar, Corning Inc., Corning, NY), and incubated at 37°C under an atmosphere of 5.0% CO_2. The medium was changed every 2 days. Only primary cultures (of 5–7 days duration) were used in the transfection experiments.

AMPLIFICATION AND PURIFICATION OF PLASMIDS CONTAINING THE β-GALACTOSIDASE, TGF-β1 OR PTHRP GENES DRIVEN BY HCMV PROMOTER/ENHANCER SEQUENCES

An expression vector for the β-galactosidase gene driven by the hCMV promoter/enhancer was obtained from Promega Inc. (Madison, WI). The TGF-β1 gene (ATCC) and the PTHrP 1-87 and 1-173 constructs[51] were inserted downstream of an hCMV promoter/enhancer in mammalian expression vectors. All plasmids were amplified and purified using standard biochemistry technique[53]. Briefly, *E. coli* (XL1-blue: Stratagene, Inc., La Jolla, CA) were transformed with the plasmids. The transformed cells were amplified, the plasmid was isolated by CsCl density centrifugation and purified by passage through an A-5M Biogel column (Biorad Inc, San Francisco, CA) as per manufacturer's protocols. Approximately 5 μg of the purified plasmid was electrophoresed through a 1% agarose gel and determined to be free of contaminating RNA or chromosomal DNAs**.**

TRANSFECTION TO TEST THE EFFICIENCY OF THE DNA/TRANSFERRIN-POLY-L-LYSINE/LIPOSOME (DTPLL) COMPLEXES

Primary perichondrial cells from rabbit ribs were cultured on tissue culture plates in media (α-MEM containing 0.5 mM L-glutamate and 500 U/ml penicillin/streptomycin and supplemented with 10% FBS), while primary chondrocytes were cultured in media consisting of DMEM/F12, 50/50, supplemented with 10% FBS and 25 μg ascorbic acid, 50 μg gentamicin/ml media at 37°C under an atmosphere of 5.0% CO_2. The cells were allowed to proliferate and achieve 70–80% confluence on the 35-mm diameter culture plate or culture slides (Costar, Corning Inc.) (i.e. 0.5×10^6 cells per plate). These cells were permeabilized with 0.00175% (w/v) lysolecithin (Sigma, St Louis, MO) for exactly 2 min. The cells were subsequently washed with 3 ml of serum free medium (SF-α-MEM). The DTPLL technique has been summarized in Fig. 1. The purified plasmid DNA (D) (4 μg) was incubated with 16 μl of 10 μg/μl stock transferrin-poly-l-lysine complex (TPL) suspended in 0.5 ml SF-α-MEM. Cationic liposome solution (24 μl) was diluted in 0.5 ml SF-α-MEM. The plasmid DNA/transferrin-poly-l-lysine complexes (DTPL) were added to the liposomes at a 1:1 ratio and allowed to form tertiary complexes for 15–20 min at room temperature. The DNA/transferrin-poly-l-lysine/liposome (DTPLL) tertiary complexes were incubated for 5 h at 37°C in serum-free medium (α-MEM) with the previously permeabilized perichondrium and cartilage derived cells under an atmosphere of 5.0% CO_2 (Protocol summarized in Fig. 1). The media was replaced with fresh media (α-MEM containing 0.5 mM L-glutamate and 500 U/ml penicillin/streptomycin and supplemented with 10% FBS).

DETERMINATION OF TRANSFECTION EFFICIENCY *IN VITRO*

The β-galactosidase gene was expressed for 48 h and tested for gene expression by β-galactosidase assay with 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) or o-nitrophenyl-β-D-galactopyranoside (ONPG) (Promega) reaction. For ONPG assay, equal amounts of the cell extract was added to the 96-well plates and incubated with 100 μl of the assay buffer as per the recommendations of the manufacturer and the absorbance was measured at 420 nm in a microplate spectrophotometer. For β-galactosidase analysis, the cells were fixed in buffer containing 0.25% v/v gluteraldehyde in 1× PBS. The fixed cells were stained with X-gal solution [0.2% X-gal (from 2% stock in DMF), 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$, $3H_2O$ and 5 mM $K_3Fe(CN)_6$ prepared in 1× PBS]. Transfected cells expressing the active β-gal enzyme turn blue. The staining step was performed at 37°C for up to 8 h. Efficiency of gene transfection in X-gal stained cells was determined as follows: five fields from each plate were viewed under the microscope. The portion of blue cells was determined as a % of total cells. The assessment was repeated for at least three plates. Permeabilized and unpermeabilized primary cells in 35 mm diameter tissue culture plates were transfected using the following reagents: (1) liposomes (24 μl)/DNA (4 μg), (2) transferrin and liposomes/DNA, (3) transferrin, poly-l-lysine (Mw=70 kDa) and liposome/DNA, (4) transferrin, poly-l-lysine (Mw=150 kDa) and liposome/DNA, and (5) poly-l-lysine covalently linked to transferrin (1:1 molar ratio) and liposome/DNA (the DTPLL complex).

DETERMINATION OF TRANSFECTION EFFICIENCY *IN VIVO*

For cell implantation studies, the β-gal gene was allowed to express for 12 h in culture and the cells were seeded into PLA scaffold according to the protocol described previously[36,37]. Standard surgical and animal care procedures, published previously, were used to create a 3 mm deep×3.7 mm diameter wide full thickness articular cartilage defect in three rabbits' femoral condyles[36,37]. The transfected cell-seeded PLA scaffold was press fitted into the defect. The knee was sutured and the rabbits were cared for using standard protocols. The femoral condyles were harvested after 1 week and the β-galactosidase activity was tested using X-gal reagent. Harvested condyles were dipped in 0.2% X-gal solution (shown above) overnight, decalcified in EDTA, fixed in Bouin's solution, embedded in paraffin and sectioned into 5 μm sections by cutting along the midsagittal plane. Five central sections per animal were analysed.

ISOLATION OF DNA-FREE RNA AND RT-PCR TO STUDY TGF-β1 GENE EXPRESSION

Total cellular RNA was purified from cells transfected with TGF-β1 containing vector or with the same vector lacking the TGF sequences (i.e. mock transfection). The cells were extracted with 4 M-guanidine thiocyanate/phenol (water saturated) as previously described[21]. To eliminate any possible carry-over genomic DNA contamination, the RNA was digested with RNase-free DNase I (RQ1-DNase I, Promega, Inc.) and extracted twice with

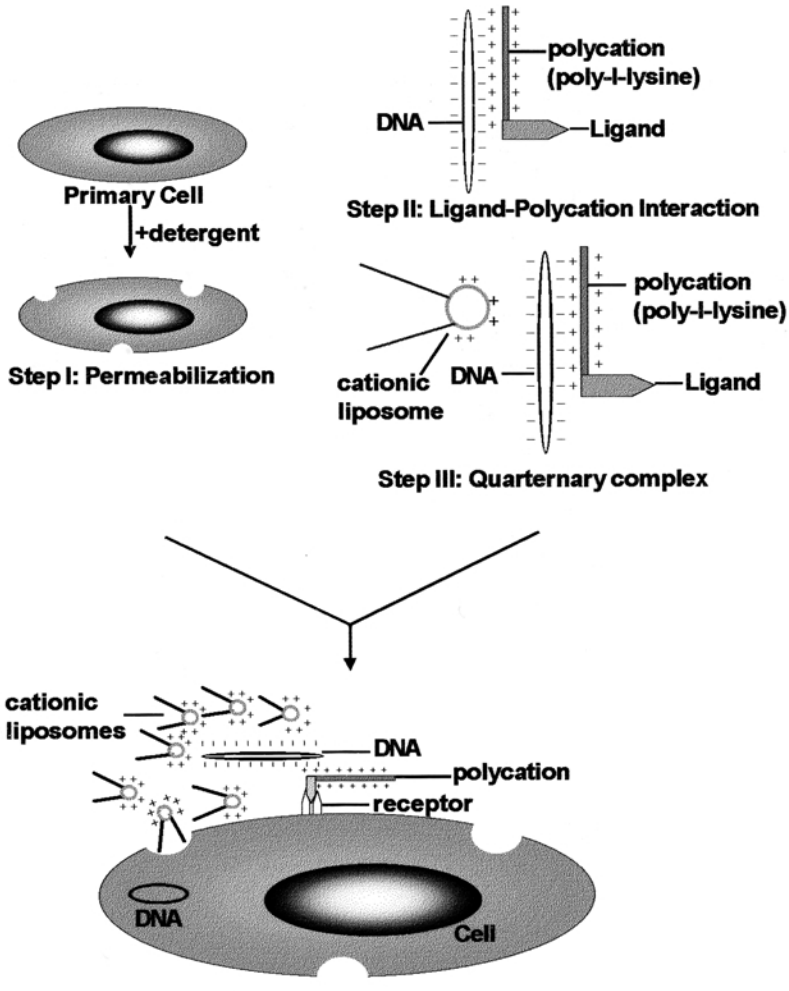

Fig. 1. Schematic description of the three-step protocol for high-efficiency transfection of primary cells. A three-step method is described. Where: D=plasmid DNA, T=transferrin, PL=poly-l-lysine, L=liposomes, DTPLL=DNA/transferrin/poly-L-lysine/liposomes complex.

phenol/chloroform/isoamyl alcohol (24:24:1 mixture) and precipitated in 70% v/v ice-cold ethanol. The RT-PCR was performed using TGF-β1 specific primers (5′-CTGGAC ACCAACTATTGCTTC-3′ 5′-CTTGCGGCCCACGTAGTA CAC-3′) and assessed as described previously[38]. As a control, RT-PCR was performed on β-actin gene. The PCR amplification was performed for 30 cycles as the mid-point of linear amplification, which was determined as follows: PCR was performed from 20 to 34 cycles. The PCR products for every other cycle (i.e. 22, 24, 26 etc.) were electrophoresed on an agarose gel and the band intensity was quantitated by image analysis of the gel scans using the NIH image analysis software (NIH image v1.6). The plot of image intensity vs cycle number was used to identify a mid-point of linear amplification.

PTHrP IMMUNOASSAYS

Twenty-four hours post-transfection the culture media was replaced with culture media containing 1% FBS (i.e. α-MEM with 1× antibiotic solution, L-glutamate and 1% FBS). PTHrP expression was studied in either conditioned media or cell extracts. Cells were pelleted and resuspended in extraction buffer (0.25 M Tris pH 7.5, 0.25% Tween-20 (supplemented with 0.1% of Triton X-100) and 0.25 mM EDTA) at a concentration of 10^6 cells/ml extraction buffer. The immunoassay was performed using rabbit antiserum to PTHrP 1-34 peptide as described previously[51].

Results

REAGENT OPTIMIZATION *IN VITRO*

In a series of experiments using different combinations of liposomes, transferrin, transferrin-poly-l-lisine conjugates and plasmids, the transfection efficiency of primary perichondrial cells and chondrocytes was optimized. Various combinations of the permeabilizing agents were also attempted. The useable range of permeabilization reagent was defined as the concentration at which minimal cell death occurred (approx. <1%) following 24 h of culture. Inclusion of transferrin in the transfection of the permeabilized cells gave efficiency of about 25%, whereas inclusion of poly-l-lysine (Mw=70 kDa) alone produced transfection efficiency of 20%. Use of unbound transferrin and poly-l-lysine (70 kDa) resulted in efficiencies as high as 40%. The use of poly-l-lysine of Mw 150 kDa (at the concentrations

Table I
Efficiency of gene transfection into permeabilized primary perichondrial cells

Treatment	Efficiency (%)
DNA+Transferrin+Liposomes	26.3±4.0
DNA+Transferrin+Poly-l-lysine+Liposomes	40.8±8.7
DNA±covalently linked Transferrin-Poly-l-lysine+Liposomes	71.1±11.6

Efficiency of expression was determined microscopically by counting β-galactosidase positive 'blue' cells.

tested) was found to be toxic to perichondrial cells. The use of DNA/Transferrin-poly-l-lysine (covanlently bonded) and cationic liposomes (DTPLL) together resulted in greater than 70% transfection efficiency (data summarized in Table I).

TRANSFECTION EFFICIENCY USING OPTIMIZED CONDITIONS *IN VITRO*

The transformation efficiency was determined by test for β-galactosidase enzyme activity by the X-gal reagent. Blue cells were indicative of β-galactosidase activity (Fig. 2). Transfection of primary rabbit perichondrial cells and chondrocytes using this method gave efficiencies of over 70% [Fig. 3(b)] as compared with the mock (plasmid lacking the β-galactosidase gene) transfection [Fig. 3(a)]. ONPG reaction (β-gal converts ONPG to o-nitrophenol (yellow) and galactose) was used to confirm the staining assay and showed that the inclusion of transferrin-poly-l-lysine (TPL) enhanced transfection efficiency into permeabilized cells significantly as measured by optical density at 420 nm (Fig. 4).

TEST OF SYSTEM EFFICACY *IN VIVO* IN A RABBIT IMPLANTATION MODEL

For *in-vivo* implantation transfected perichondrial cells were seeded into a PLA scaffold 24 h post-transfection and placed into a 3×3.7 mm defect in the rabbit femoral condyles. The condyles were harvested 1 week post-implantation and assessed qualitatively for β-gal enzyme activity. Cells in the surface layer of the implant were found to be positive for β-galactosidase gene activity harvested 1 week post-implantation (Fig. 5), implying that the seeded cells continued to express the transfected gene.

Primary perichondrial cells transfected with TGF-β1 expression vector showed increased mRNAs for TGF-β1 by semi-quantitative RT-PCR (Fig. 6). Primary chondrocytes were transfected with PTHrP genes 1-87 and 1-173 expression constructs and resulted in increased PTHrP gene expression (Fig. 7) measured using the anti-PTHrP 1-34 antibodies. The cartilage cells demonstrated both secretion and storage of PTHrP in the non-transfected, basal state and over expression of PTHrP 1-87 from the CMV promoter-driven PTHrP 1-87 plasmid after transfection (approximately 10-fold above basal level in the media and over two-fold in the cell extract). Transfection with PTHrP 1-173 plasmid did not show over-expression of PTHrP in the media [Fig. 7(a)] but did demonstrate a 50% increase in the level of PTHrP in the cell extract. These transfected cells continued to express PTHrP for at least 13 days post-transfection (data not shown). These results are consistent with the observation that, elimination of the carboxy-terminal sequences of PTHrP 1-173 (i.e. 1-87 construct) increased the production and secretion of the truncated form[51].

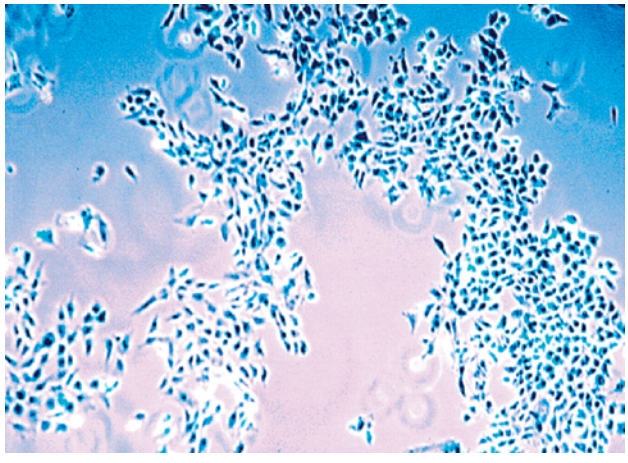

Fig. 2. Transfection of primary perichondrium derived cells by the DTPLL method outlined in Fig. 1. An example of a microscopic field of the X-gal-stained cells. Cells were transfected at 70–80% confluence, allowed to grow to for 48 h, and assayed for enzyme expression by X-gal. β-Galactosidase expression monitored by *in situ* X-gal staining (blue cells)—most cells in this field were labeled blue.

Discussion

The efficacy of the protocol described here could be attributed to the following sequence of events: (1) permeabilization: the cell membrane was made slightly porous prior to the introduction of genes into the cells with the help of a mild detergent (lysolecithin), (2) ligand/polycationic complex: a specific ligand (transferrin) was

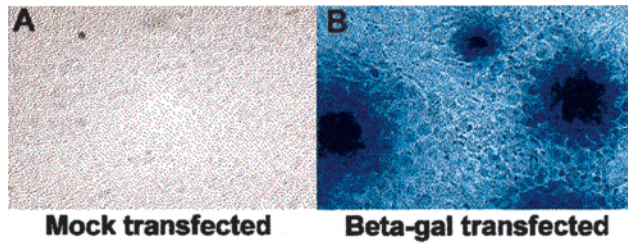

Fig. 3. A representative microscopic field showing high-efficiency transfection of primary chondrocytes for β-gal activity by *in-situ* X-gal staining (blue cells). (A) Mock transfection with the CMV plasmid lacking the β-gal gene sequences. (B) Transfection with the β-gal plasmid as described in Methods. The foci are present in normal confluent cultures of primary chondrocytes but are visualized when cells are stained (B).

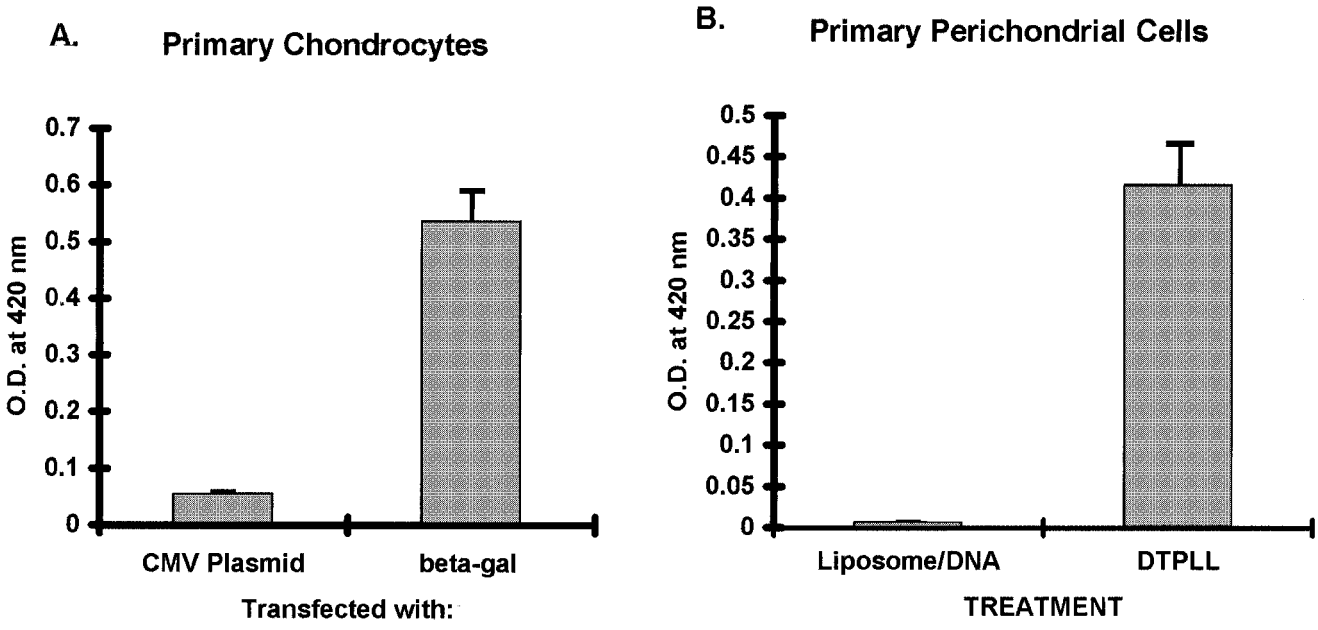

Fig. 4. Assessment of relative transfection efficiency by ONPG assay. Equal amounts of the cell extracts of transfected perichondrium (A) transfected using liposomes alone or the DTPLL method and cartilage cells (B) either mock transfected or transfected with the marker (β-gal expression plasmid) were analysed by the ONPG assay described in Methods. Relative β-gal activity was assessed for differences in optical density (OD) at 420 nm.

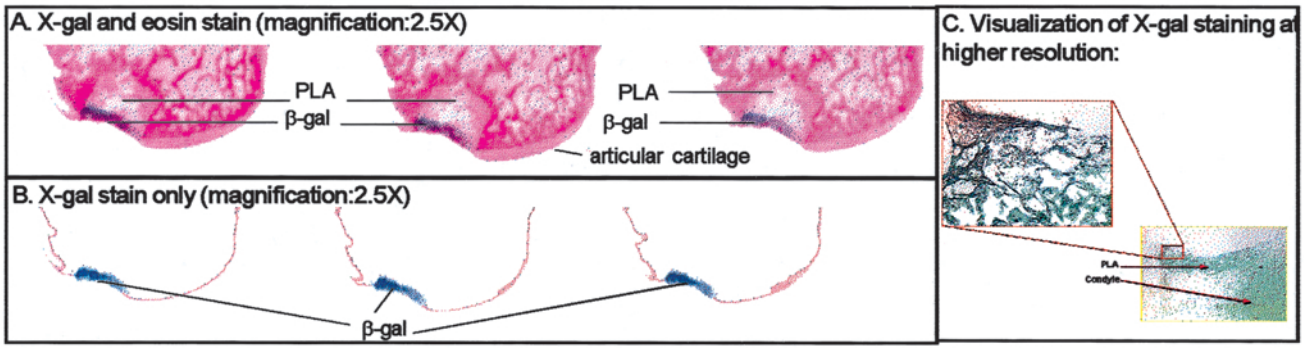

Fig. 5. *In-vivo* assessment of transfected primary perichondrial cells. Cell seeded PLA cores were seeded into experimentally created osteochondral defects in the rabbit femoral condyles. Assessment of β-gal enzymatic activity by X-gal 1 week post-implantation. (A) X-gal and eosin stained sections. (B) Staining with the X-gal solution alone. (C) High resolution image of a representative X-gal stained section.

covalently attached to a polycationic core (poly-l-lysine) to create a scaffold to facilitate ionic interaction with negatively charged DNA, (3) ligand/receptor interaction: the DNA/ligand-poly-l-lysine-complex was delivered very close to the cell due to the binding of the ligand to its receptor (i.e. the receptor ligand interaction), and (4) cationic liposome mediated delivery: the DNA is putatively delivered into the cell by hydrophobic interactions between the cationic liposomes[20] and the cell membrane that had been previously permeabilized.

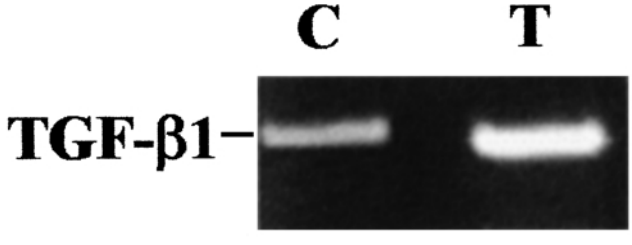

Fig. 6. Analysis of TGF-β1 in transfected primary perichondrial cells by RT-PCR. RT-PCR products were electrophoresed on an agarose gel and visualized by ethidium bromide intercalation. Expected sized products (495 bps) were observed for the control and TGF-β1 (T) transfected cells. The β-actin gene expression did not vary between control (C) and TGF-β1 transfected (T) cells.

This gene delivery protocol has shown efficacy *in vitro* and *in vivo* in an animal model. *In vitro*, more than 70% of primary perichondrial cells and chondrocytes were determined to be positive for the transfected gene by X-gal staining (Figs 2 and 3) and relative reaction with ONPG (Fig. 4). These cells were implanted in the rabbit knee into an experimentally created osteochondral defect. After 1 week the defect contained cells that continued to express the transfected gene. The staining was primarily observed on the top portion (cartilage surface) of the implanted cells (Fig. 5). This observation implies the possibility that cells in the inner core of the implanted scaffold are unable to express any active β-gal enzyme,

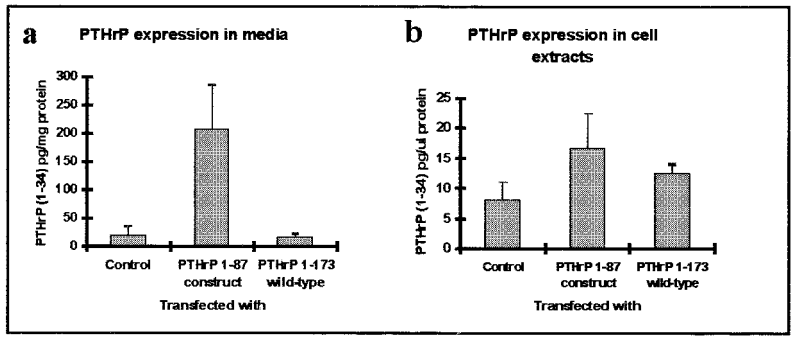

Fig. 7. PTHrP protein expression in transfected primary chondrocytes: PTHrP levels were assessed by immunoassays using anti-PTHrP (1-34 peptide) antibody in (A) culture media and (B) cell extracts 3 days post-transfection.

suggesting, the possible problem of nutrient permeability in this size scaffold following 1 week of *in-vivo* implantation.

The expression of the 1-87 truncated PTHrP construct in the media fraction was several fold larger than its wild-type isomer 1-173 in transfected primary chondrocyte cultures, while, expression in the cellular fraction was about two-fold as large (Fig. 7). These relative differences are consistent with the observation that elimination of the carboxy-terminal sequences of PTHrP 1-173 increases production and secretion of the truncated 1-87 isomer[51]. Primary perichondrial and chondrocytic cells transfected with TGF-β1 and PTHrP form the first part of the studies to show physiological growth and regulatory changes, such as changes in cell proliferation and gene expression.

The DTPLL transfection system is envisioned to be generally applicable for gene therapy using stem cells from the perichondrium, periosteum or chondrocytes[54]. Introduction of morphoregulatory factors such as TGF-β1[38,55–58], PTHrP[43,44,47], connective tissue growth factor (CTGF) and bone morphogenetic proteins (i.e. BMP-2)[57,58] and transcription factors (e.g. SOX-9)[59,60] may enhance chondrogenic phenotype of these cells or may lead to the development of more physiologically relevant cell culture models for functional studies. In previous studies, primary cultured perichondrium, bone marrow, periosteum and cartilage derived cells have been shown to partially enhance repair of osteochondral defects in animal models[34,36,37,54]. The duration of expression of the transfected gene has been noted here for the entire study period of 1 week in the animal model. The gene expression following this period remains to be determined. The phenotypic changes induced by the transfected cells in this time period may, however, be sufficient to enhance cartilage repair. We anticipate that in the near future spatially and temporally restricted over-expression of morphoregulatory factors or transcription factors in primary autologous cells and subsequent implantation of these in cell-seeded cores (such as the PLA) may form a useful therapeutic modality for tissue engineering.

Acknowledgments

This work was supported by University of California Academic Senate Grant, NIH grants: AR28467, POIAG07996, a VA Merit Review Award and an Arthritis Foundation Biomedical Sciences Research Award. We would like to acknowledge the expert technical assistance provided by Doug Burton and Karen Bowden.

Abbreviations

β-gal, β-galactosidase; hCMV, human cytomegalovirus; DTPL, DNA/transferrin-poly-l-lysine; DTPLL, DNA/transferrin-poly-l-lysine/liposomes; TGF-β1, transforming growth factor-β1; PTH, parathyroid hormone; PTHrP, parathyroid hormone related peptide; Ihh, Indian hedgehog; PLA, polylactic acid; BMP, bone morphogenetic protein; X-gal, 5-bromo-4-chloro-3-indoyl-β-D-galactoside; ONPG, o-nitrophenyl-β-D-galactopyranoside; DMF, dimethyl formamide; RT-PCR, reverse transcription-polymerase chain reaction; CTGF, connective tissue growth factor.

References

1. Versland MR, Wu CH, Wu GY. Strategies for gene therapy in the liver. Sem Liver Dis 1992;12:332–9.
2. Dematteo RP, Raper SE, Ahn M, Fisher KJ, Burke C, Radu A, *et al*. Gene transfer to the thymus. A means of abrogating the immune response to recombinant adenovirus. Ann Surg 1995;222:229–42.
3. Ghivizzani SC, Lechman ER, Kang R, Tio C, Kolls J, Evans CH, *et al*. Direct adenovirus-mediated gene transfer of interleukin 1 and tumor necrosis factor alpha soluble receptors to rabbit knees with experimental arthritis has local and distal anti-arthritic effects. Proc Natl Acad Sci USA 1998;95:4613–8.
4. Baragi VM, Renkiewicz RR, Jordan H, Bonadio J, Hartman JW, Roessler BJ. Transplantation of transduced chondrocytes protects articular cartilage from Interleukin 1 induced extracellular matrix degradation. J Clin Invest 1995;96:2454–60.
5. Alden TD, Pittman DD, Hankins GR, Beres EJ, Engh JA, Das S, *et al.* In vivo endochondral bone formation using a bone morphogenetic protein 2 adenoviral vector. Hum Gene Ther 1999;10:2245–53.
6. Bessis N, Boissier MC, Ferrara P, Blankenstein T, Fradelizi D, Fournier C. Attenuation of collagen-induced arthritis in mice by treatment with vector cells engineered to secrete interleukin-13. Euro J Immun 1996;26:2399–403.
7. Doherty PJ, Zhang H, Tremblay L, Manolopoulos V, Marshall KW. Resurfacing of articular cartilage explants with genetically-modified human chondrocytes in vitro. Osteoarthritis Cartilage 1998;6: 153–9.
8. Evans CH, Ghivizzani SC, Kang R, Muzzonigro T, Wasko MC, Herndon JH, *et al*. Gene therapy for rheumatic diseases. Arthritis Rheum 1999;42:1–16.

9. Ginsburg HS, Moldawer LL, Schgal PB, Redimgton M, Kilian DL, Chanock RM, *et al*. A mouse model for investigating the molecular pathogenesis of adenovirus pneumonia. Proc Natl Acad Sci USA 1991;88: 1651–5.
10. Yang Y, Li Q, Ertl HCJ, Wilson JM. Cellular and humoral immune responses to viral antigens create barriers to lung directed gene therapy with recombinant adenoviruses. J Virol 1995;69:2004–15.
11. Yang Y, Su Q, Wilson JM. Role of viral antigens in destructive cellular immune responses to adenovirus vector-transduced cells in mouse lungs. J Virol 1996;70:7209–12.
12. Yei S, Mittereder N, Tany K, O'Sullivan C, Trapnell BC. Adenovirus-mediated gene transfer for cystic fibrosis: quantitative evaluation of repeated in vivo vector administration in the lung. Gene Ther 1994;1: 192–200.
13. McCray PB, Wang G, Kline JN, Zabner J, Chada S, Jolly DJ, *et al*. Alveolar macrophages inhibit retrovirus-mediated gene transfer to airway epithelia. Hum Gene Ther 1997;8:1087–93.
14. Trapnell BC, Gorziglia M. Gene therapy using adenoviral vectors. Curr Opin Biotechnol 1994;5: 617–25.
15. Sokol DL, Gewirtz AM. Gene therapy: Basic concepts and recent advances. Crit Rev Euk Gene Expr 1996;6:29–57.
16. Colledge WH. Cystic fibrosis gene therapy. Curr Opin Gene Dev 1994;4:466–71.
17. Fairbarn LJ, Cross MA, Arrand JR. Patterson Symposium 1993—Gene Therapy. Br J Cancer 1994;59:972–5.
18. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, et al. Lipofection: A highly efficient, lipid-mediated DNA transfection procedure. Proc Natl Acad Sci USA 1987;84:7413–17.
19. Felgner PL, Ringold GM. Cationic liposome-mediated transfection. Nature 1989;337:387–8.
20. Wheeler CJ, Felgner PL, Tsai YJ, Marshall J, Sukhu L, Doh SG, *et al*. A novel cationic lipid greatly enhances plasmid DNA delivery and expression in mouse lung. Proc Natl Acad Sci USA 1996;93:11454–9.
21. Goomer RS, Holst BD, Wood IC, Jones FS, Edelman GM. Regulation in vitro of an L-CAM enhancer by homeobox genes HOXD9 and HNF-1. Proc Natl Acad Sci USA 1994;91:7985–9.
22. Brant WO, Goomer RS, Amiel D. Assessment of liposome-mediated transfectional efficacy of aged human chondroprogenitor cells. Am Fed Med Res (AFCR) Mtng, Feb 5-8 1997;45:159A.
23. Cooper MJ. Noninfectious gene transfer and expression systems for cancer gene therapy. Sem Oncol 1996;23:172–87.
24. Terkeltaub RJ. The immortalized chondrocyte: art without sacrifice. J Clin Invest 1994;94:2172–3.
25. Goldring MB, Birkhead JR, Suen LF, Yamin R, Mizuno S, Glowacki J, *et al*. Interleukin-1 beta-modulated gene expression in immortalized human chondrocytes. J Clin Invest 1994;94(6):2307–16.
26. Greenleaf WJ, Bolander ME, Sarkar G, Goldring MB, Greenleaf JF. Artificial cavitation nuclei significantly enhance acoustically induced cell transfection. Ultrasound Med Biol 1998;24:587–95.
27. Canonico AE, Plitman JD, Coanry JT, Mayrick BO, Brigham KL. No lung toxicity after aerosol or intravenous delivery of plasmid-cationic liposome complexes. J Appl Physiol 1994;77:415–19.
28. Wagner E, Zenke M, Cotten M, Beug H, Birnstiel ML. Transferrin-polycation conjugates as carriers for DNA uptake into cells. Proc Natl Acad Sci USA 1990;87: 3410–14.
29. Taxman DJ, Lee ES, Wojchowski DM. Receptor-targeted transfection using stable Maleimido-Transferrin/Thio-Poly-l-lysine conjugates. Analytical Biochem 1993;213:97–103.
30. Lee RJ, Huang L. Folate-targeted, anionic liposome entrapped polylysine-condensed DNA for tumor cell-specific gene transfer. J Biol Chem 1996;271: 8481–7.
31. Mack KD, Walzem RL, Lehmann-Bruinsma K, Powell JS, Zeldis JB. Polylysine enhances cationic liposome-mediated transfection of hepatoblastoma cell line Hep G2. Biotech Appl Biochem 1996; 23(Pt 3):217–20.
32. Gao X, Huang L. Potentiation of cationic liposome-mediated gene delivery by polycations. Biochemistry 1995;35:1027–36.
33. Cheng PW. Receptor ligand-facilitated gene transfer: Enhancement of liposome mediated gene transfer and expression by transferrin. Hum Gene Ther 1996;7:275–82.
34. Wakatani S, Goto T, Pineda SJ, Young RG, Mansour JM, Caplan AI, et al. Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. J Bone Joint Surg 1994;76:579–92.
35. Akentijevich I, Pastan I, Lunardi-Iskander Y, Gallo R, Gottesman MM, Thierry AR. In vitro and in vivo liposome-mediated gene transfer leads to MDR1 expression in mouse bone marrow progenitor cells. Hum Gene Ther 1996;7:1111–22.
36. Chu CR, Dounchis JS, Yoshioka M, Sah RL, Coutts RD, Amiel D. Osteochondral repair using perichondrial cells. A 1 year study. Clin Orthop Rel Res 1997;340:220–9.
37. Chu CR, Coutts RD, Yoshioka M, Harwood FL, Monosov AZ, Amiel D. Articular cartilage repair using allogeneic perichondrocyte-seeded biodegradable porous polylactic acid (PLA): a tissue-engineering study. J Biomed Mat Res 1995;29:1147–54.
38. Dounchis JS, Goomer RS, Harwood FL, Khatod M, Coutts RD, Amiel D. Chondrogenic phenotype of perichondrium-derived chondroprogenitor cells is influenced by transforming growth factor-beta 1. J Orthop Res 1997;15:803–7.
39. Moseley JM, Hayman JA, Danks JA, Alcorn D, Grill V, Southby J, et al. Immunohistochemical detection of parathyroid hormone-related protein in human fetal epithelia. J Clin Endo Metab 1991;73:478–84.
40. Suda N. Parathyroid hormone-related protein (PTHrP) as a regulating factor of endochondral bone formation. Oral Dis 1997;3:229–31.
41. Yamazaki K, Suda N, Kuroda T. Immunohistochemical localization of parathyroid hormone-related protein in developing mouse Meckel's cartilage and mandible. Archs Oral Biol 1997;42:787–94.
42. Amizuka N, Karaplis AC, Henderson JE, Warshawsky H, Lipman ML, Matsuki Y, et al. Haploinsufficiency of parathyroid hormone-related peptide (PTHrP) results in abnormal postnatal bone development. Dev Biol 1996;175:166–76.
43. Tsukazaki T, Ohtsuru A, Enomoto H, Yano H, Motomura K, Ito M, *et al*. Expression of parathyroid

hormone-related protein in rat articular cartilage. Calcif Tissue Inter 1995;57:196–200.
44. Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science 1996;273(5275):613–22.
45. Lee K, Deeds JD, Bond AT, Jüppner H, Abou-Samra AB, Segre GV. In situ localization of PTH/PTHrP receptor mRNA in the bone of fetal and young rats. Bone 1993;14:341–5.
46. Lee K, Deeds JD, Chiba S, Un-No M, Bond AT, Segre GV. Parathyroid hormone induces sequential c-fos expression in bone cells in vivo: in situ localization of its receptor and c-fos messenger ribonucleic acids. Endocrinology 1994;134:441–50.
47. Amizuka N, Warshawsky H, Henderson JE, Goltzman D, Karaplis AC. Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. J Cell Biol 1994;126:1611–23.
48. Karaplis AC, Luz A, Glowacki J, Bronson RT, Tybulewicz VL, Kronenberg HM, *et al*. Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. Genes Dev 1994; 8:277–89.
49. Merryman JI, DeWille JW, Werkmeister JR, Capen CC, Rosol TJ. Effects of transforming growth factor-beta on parathyroid hormone-related protein production and ribonucleic acid expression by a squamous carcinoma cell line in vitro. Endocrinology 1994;134: 2424–30.
50. Amling M, Neff L, Tanaka S, Inoue D, Kuida K, Weir E, *et al*. Bcl-2 lies downstream of parathyroid hormone-related peptide in a signaling pathway that regulates chondrocyte maturation during skeletal development. J Cell Biol 1997;136:205–13.
51. Ditmer LS, Burton DW, Deftos LJ. Elimination of the carboxy-terminal sequences of parathyroid hormone-related protein 1-173 increases production and secretion of the truncated forms. Endocrinology 1996;137:1608–17.
52. Häuselmann HJ, Aydelotte MB, Schumacher BL, Kuettner KE, Gitelis SH, Thonar EJ. Synthesis and turnover of proteoglycans by human and bovine adult articular chondrocytes cultured in alginate beads. Matrix 1992;12:116–29.
53. Sambrook J, Fritsch E, Maniatis T (Eds). Molecular Cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Press 1989:1.3–1.105.
54. Kang R, Marui T, Ghivizzari SC, Nita IM, Georgescu HI, Suh JK, *et al*. Ex vivo gene transfer to chondrocytes in full-thickness articular cartilage defects: a feasibility study. Osteoarthritis Cart 1997;5:139–43.
55. Ballock RT, Heydemann A, Wakefield LM, Flanders KC, Roberts AB, Sporn MB. TGF-β1 prevents hypertrophy of epiphyseal chondrocytes: regulation of gene expression for cartilage matrix protein metalloproteases. Dev Biol 1993;158:414–29.
56. Galera P, Vivien D, Pronost S, Bonaventura J, Penfornis H, Loyau G, *et al*. Effect of transforming growth factor-β1 (TGF-β1) on matrix synthesis by monolayer cultures of rabbit articular chondrocytes during the dedifferentiation of process. Exp Cell Res 1992;200: 379–92.
57. Roark E, Greer K. Transforming growth factor-β and bone morphogenetic protein-2 act by distinct mechanisms to promote chick limb cartilage differentiation in vitro. Dev Dynamics 1994;200:103–16.
58. Luyten FP, Chen P, Paralkar V, Reddi AH. Recombinant bone morphogenetic protein-4, transforming growth factor-β1, and activin A enhance the cartilage phenotype of articular cartilage chondrocytes in vitro. Exp Cell Res 1994;210:224–9.
59. Lefebvre V, Hung W, Hardy VR, Goodfellow PN, de Crombugghe B. SOX9 is a potent activator of the chondrocyte-specific enhancer of the ProαI(II) collagen gene. Mol Cell Biol 1997;17:2336–46.
60. Bell DM, Leung KK, Wheatley SC, Ng LJ, Zhou S, Ling KW, *et al*. SOX9 directly regulates the type-II collagen gene. Nature Gen 1997;16:174–8.